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Fungal Endophytes: Australian Terrestrial Orchids

Shalika Mehra

Abstract

Orchids are unique as they lack a functional rooting system and share an obligate relationship with their fungal symbionts. This relationship supports their host's nutritional demands from seed germination to its later development. The orchid fungal endophytes explore large areas in the soil as, to which orchid roots have no access, and thus acquire both organic and inorganic nutrients beyond the depletion zone at low carbon cost. Both 'autotrophic' (green) and 'mycoheterotrophic' species occur in the Orchidaceae, but the term 'mixotrophic' is possibly a truer description of the carbon economy of many green orchids. Some of the major ecological threats of an Australian landscape are habitat destruction and fragmentation. There is little known about the nutritional sources and saprophytic ability of orchid mycorrhizal fungi (OMF) and their role in providing nutrition to orchids. However, several integrated approaches have been developed for the conservation, management and restoration of these plants in wild but there is an urgent need to set appropriate conservation priorities to prevent the loss of habitats for these endangered species in terms of their fungal endophytes. This chapter focuses on the protection of these endangered Australian orchid species by understanding the nutritional behavior of their endophytes.

Keywords: orchid mycorrhizal fungi (OMF), autotrophic, endangered, conservation, mycoheterotrophic (MH)

1. Introduction

Orchids (family Orchidaceae) being iconic are at the front line of extinction, with 17,000–35,000 species distributed globally and are under threat [1–3]. The family is cosmopolitan in its distribution, but the genera and species are highly endemic [4]. In the Orchidaceae, greater levels of ecological specializations associated with global climate change, have a direct impact on the species diversity and levels of threat, to the extent that many terrestrial orchids in temperate regions have become extinct.

Australia is rich in terrestrial orchid diversity (82%) with approximately 115 genera. The Southwest Australia Floristic region (SWAFR) is among 25 hotspots of biodiversity globally [5]. They can be found in a wide range of habitats across the continent and are usually categorized as epiphytes, lithophytes, and terrestrials, where epiphytes and lithophytes are mostly distributed in the warm and moist regions of tropics (18%) while few species are found in temperate regions of eastern Victoria and Tasmania [5]. They are mostly found in sclerophyll open forests and swampy coastal scrub lands. They grow on the ground especially in open habitats

such as grasslands, heathlands and forest floors with low annual rainfall, showing seasonal changes and are mostly distributed in the southern temperate zones of Australia which have a Mediterranean climate. Most of the orchids growing in these temperate regions are deciduous, surviving climate extremes beneath the soil surface by undergoing dormancy [6].

They usually have subterranean fleshy thick tubers or tuberoids that store nutrients during dormancy. Some of the most common terrestrial orchid genera found in Australia are, *Caladenia* (Spider orchids), *Pterostylis* (Greenhoods), *Diuris* (Donkey orchid), *Acianthus* (Mosquito orchid), *Prasophyllum*, *Thelymitra* (Sun orchids), *Microtis* and *Glossodia* (**Figure 1**) [5]. *Caladenia*'s are (spider orchids) endemic to Australia and represent one of the extraordinary terrestrial orchids with a large number of threatened and rare taxa [6]. In total there are 132 species of spider orchids which are mostly distributed throughout southern Australia.

From the ecological point of view, these orchids could act as ecological indicators of a healthy environment [7]. Due to their complex interactions with pollinators, fungal endophytes, and associated host trees, their conservation involves challenges at species-specific levels. These challenges are mostly linked to their habitat destruction and fragmentation, land use, climate change and unsustainable exploitation of biodiversity [8, 9]. Also, most of the terrestrial orchids of Australia, are continuously encountered by inappropriate fire regimes at different developmental stages of its life cycle, and places 74% of threatened orchid species at risk

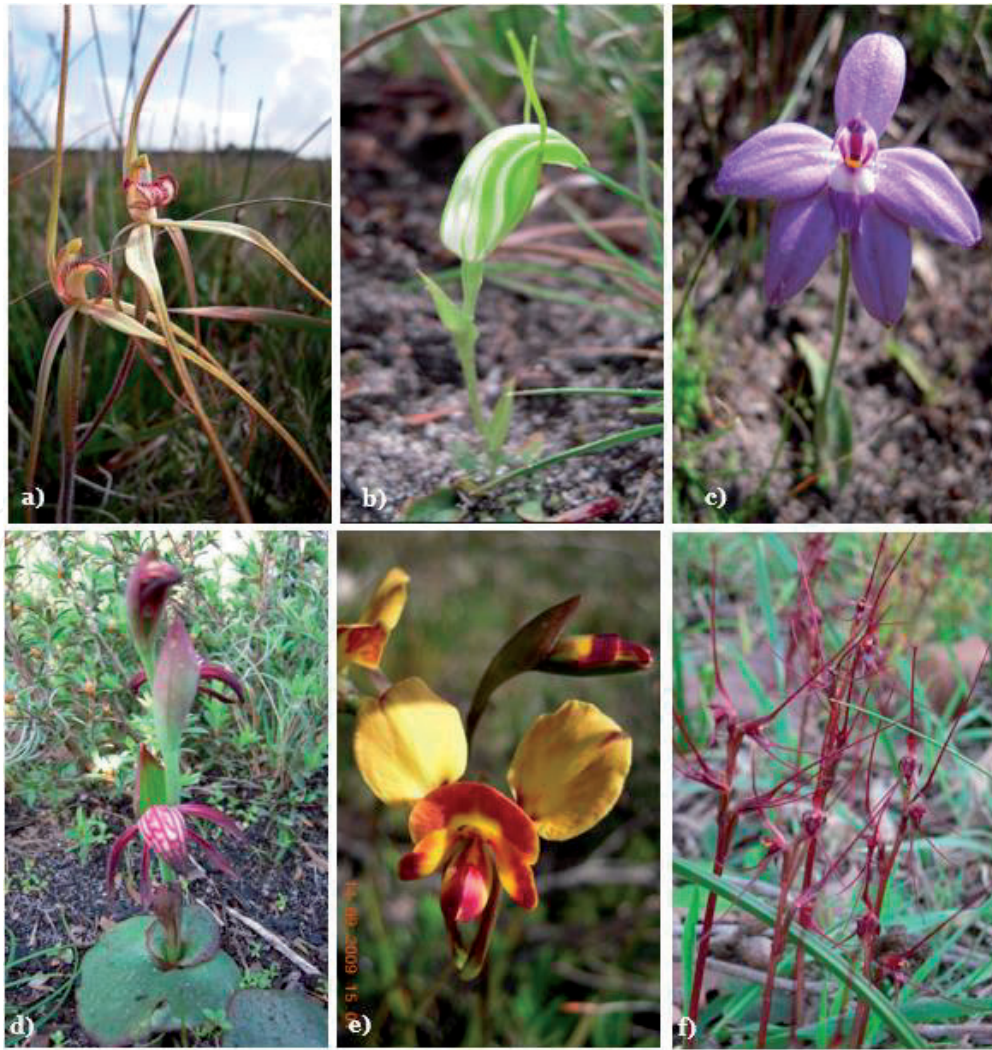


Figure 1. Australian terrestrial orchid species, (a) *Caladenia* spp. (b) *Pterostylis* spp. (c) *Glossodia* spp. (d) *Corybas* spp. (e) *Diuris* spp. (f) *Acianthus* spp.

of extinction [10–12]. Recently, the impact of nature-based tourism has also been reported as a major threat to the decline of threatened orchid populations in the wild in South Australia [10]. Due to these factors, the survival of various species from this genus is at risk and thus considerable effort is required from scientists and conservation practitioners to overcome these challenges of the twenty-first century. However, with the ability to use current novel technologies in orchid biology greater than ever before, we can help them conserve for future generations.

Australian soils are generally deficient in nutrients, which have mostly leached out of the sandy soils (podzols) over many millions of years [13]. In a fire-prone Australian ecosystem, fungi can have a major influence on surrounding biota and play an essential role in maintaining the healthy ecosystems as effective symbiotic partners, decomposers, nutrient cyclers and are a source of food for various organisms. The top horizon of organic matter is the major source of carbon (C), nitrogen (N) and phosphorus (P). In most coarse-rooted plants like orchids, with a poorly developed root system, mineral nutrition is highly dependent on mycorrhizal uptake of essential elements such as N and P from their surroundings [14]. Orchid mycorrhizal fungi (OMF) present in these nutrient-depleted soils are likely to derive their nutrition from the organic matter (dead roots, exoskeletons, leaves and wood in a litter), which holds various types of complex compounds. These complex molecules are further degraded into simpler forms by the activity of these mycorrhizal fungi and other microorganisms. C is usually available in complex forms such as cellulose, hemicelluloses, pectin and lignin, as well as simple soluble breakdown products from these complex polymers. Also, availability of N is usually in the form of organic peptides, proteins and amino acids and as inorganic ammonium and nitrate ions whereas phosphorus is mostly available as organic compounds such as phytic acid and sparsely available as inorganic ions such as PO_4^{3-} , HPO_4^{2-} and H_2PO_4 .

The ability of OMF to assimilate various C, N and P compounds as compared to other ericoid mycorrhizal (ERM) and ectomycorrhizal (ECM) fungi, has been studied previously but information available until now is fragmentary [15–18]. It is very important to understand the nutritional physiology of endophytes associated with terrestrial orchid species while considering any recovery plans for propagation, management, conservation and restoration of Australian endangered orchid species in wild. Therefore, in this chapter, we have discussed in general about orchid endophytes and their saprophytic ability in digesting complex resources, confined to its litter prone, open and well-drained podzol sites.

2. Orchid fungal endophytes

All orchids share obligate relationships with their endophytes, from early seed germination stages to later development of seedlings and mature plants. Endophytes are commonly found inside the healthy tissues of orchid roots as bacterial and fungal endophytes without causing any symptoms of a disease. In this mutualism, fungus provides water and mineral nutrition to the host plant which in turn provides photosynthetically fixed carbon back to its fungal partner [4], phenomenon which is commonly found in fully autotrophic orchid species [19] as compared to completely mycoheterotrophic (MH) and partially MH orchid (Mixotrophic) species [20–22].

Physiology of orchid seed germination is one of the interesting phenomena of nature and therefore must enter symbiotic interaction with a species-specific symbiont for appropriate germination. All orchid species are MH in their early stages of seed development, where orchids obtain their nutrition in the form of minerals, salts,

water and carbon supply from their fungal symbionts at least in their initial seed germination stages [23]. Once the fungus invades the minute orchid seeds (having low endosperm reserves) it kicks starts the germination process, eventually giving rise to an undifferentiated mass of cells known as protocorms. However, this mutual symbiosis between the host and its fungal partner has not been understood completely, it seems that orchid is having a complete control over-regulating the degree and level of these associations. Germination and vegetative propagation in their natural environment is very slow with a rate of <5% [24]. The distribution of orchids and their diversity is dependent on the availability of their fungal symbionts and thus understanding orchid mycorrhizal symbiosis is a key factor to conserve orchids.

2.1 Orchid symbionts: rhizoctonias and other mycorrhizal fungi

Various orchid species have heterobasidiomycetes as their symbionts [25]. The complex assemblage of fungi associated with orchids consists of Agaricomycetes (=Hymenomycetes) taxa [26]. OMF was traditionally classified as anamorphic form-genus (imperfect stage) *Rhizoctonia* (=Epulorhiza). These correspond to three distantly related basidiomycetous lineages forming teleomorphic genera, including Ceratobasidiaceae, Tulasnellaceae and Serendipitaceae [27]. Although the OMF is well known for its saprophytic abilities [4] they may be found widely as endophytes in non-orchid roots [28] without forming any symptoms of infection.

Recently, a range of mycorrhizal fungi has been found associated with different orchid species, apart from their long evolutionary history of associations with rhizoctonias [26]. OMF studies on MH and mixotrophic orchid species have shown a huge diversity of ectomycorrhizal fungi [23], including saprotrophic fungi from Mycenaceae and Psathyrellaceae and some ascomycete taxa, which suggests that depending upon their host, same fungi could have a potential to form dual associations in nature. Photosynthetic orchids can also associate with a variety of taxa, including Psathyrellaceae and saprotrophic fungal species [29].

Members of Tulasnellaceae, Serendipitaceae (Sebacinales clade B) and Ceratobasidiaceae are well known for their endophytic [30] and saprophytic abilities [31] with few exceptions from Ceratobasidiaceae where some species are plant-parasitic [27]. *Serendipita indica* is one of the well-studied, root endophyte models and is indeed found mycorrhizal with orchid roots [32]. Fungi in the Serendipitaceae are involved in a wide range of mycorrhizal associations such as ectomycorrhizas, ericoid mycorrhizas, orchid mycorrhizas and even liverworts (Jungermannoid mycorrhizas) [26, 33–35]. Phylogenetically the Serendipitaceae (formerly called Order Sebacinales) is grouped into two clades: A and B [36]. Clade A species constitutes jelly fungi, having a saprophytic ability through which they can obtain their nutritional demands from wood and other surrounding litter present in their habitat, while Clade B species are common endophytes of underground plant organs [37]. The fungi from Clade B are usually associated with orchids, for example, *Caladenia* species in Australia, are also associated with ericoid roots, though without having any proof of functional symbiosis so far [33]. There are studies which have shown presence of basidiomycetous hyphae with septal pores on and in sections of ericoid plants by transmission electron microscopy (TEM) whereas, there is an evidence of DNA sequences from the nuclear ribosomal internal transcribed spacer (ITS) region from ericoid roots that grouped within *Serendipita* group B and contained identical sequences to those from *Serendipita vermifera* isolates from Australian green orchids [4]. *S. vermifera* [30] in group B [38], has a confirmed mycorrhizal relationship with some green orchids, e.g. in *Caladenia* and *Glossodia* species and is the most common OMF found associated to these taxa [39–42].

2.1.1 Fungal identification

Traditional approaches were commonly used to identify these fungal endophytes of orchids by isolating the pelotons from the orchid tissues and maintaining them as pure cultures. Mycelia are mostly present as anamorphs and the orchid endophytes are commonly identified based on their morphological (hyphal walls), anatomical differences (spore formation and nucleus number) and anastomosis behavior [43] by using optical, scanning and electron microscopy. Most form chains of small ovoid-globular monilioid cells. Recently, several molecular approaches are extensively used to delimit the fungal endophytes of orchids (*Ceratobasidium*, *Tulasnella* and *Rhizoctonia* = *Serendipita*) which are well known for their poor taxonomy [44, 45].

2.1.1.1 Asexual stages

Rhizoctonia is remarkable in some characteristics as they branch out at acute angles when young but at right angles to the main axis at maturity, mainly constricting at the point of branching [46]. Fungi grown from pelotons usually form ovoid monilioid cells without having any clamp connections or conidia in a culture that limits their identification through morphological methods [40]. *Rhizoctonia*, is traditionally characterized on the basis of anastomosis groupings including the pathogenic strains [47]. *Rhizoctonia* species are separated on the basis of the ultrastructure of the number of nuclei in each cell and the septa, and on the basis of what can be categorized as uninucleate, binucleate or multinucleate [48]. The commonly isolated *Rhizoctonia* fungi from terrestrial orchid species are within the anamorphic genera *Ceratorhiza*, *Moniliopsis*, *Thanatephorus* and *Epulorhiza*.

2.1.1.2 Sexual stages

The commonly isolated *Rhizoctonia* fungi from terrestrial orchids are species within the teleomorphic genera, *Ceratobasidium*, *Tulasnella* and *Serendipita*. Imperfect stages of *Rhizoctonia* are commonly found in various chlorophyllous orchids. For most Australian green orchids, *in-vitro* cultures produce only monilioid cells but Warcup and Talbot obtained teleomorphic stages on *Rhizoctonia* isolates in culture [40, 41, 49], an achievement not replicated by many researchers despite numerous attempts. Because of this, the systematics of *Rhizoctonia*-type OMF has been studied using both morphological [43, 46] and molecular approaches [25, 38, 44, 45, 50], which have suggested various anamorphs and teleomorphs for this polyphyletic group.

3. Fungal endophytes of myco-heterotrophic (MH) and autotrophic orchids

The nutrition of orchids is closely tied to the nutrition of their basidiomycetous OMF. The fungal symbionts provide essential nutrients for the establishment of orchid seedlings from obligate MH stage to mixotrophic to fully autotrophic stages of their development. They can obtain their nutrition as saprophytes, by breaking down wood and other litter in their habitats or by tripartite symbiosis, in which the OMF is also ectomycorrhizal on the roots of the surrounding higher plants. Both result in networks of hyphae linking the host plants to various habitats.

In general, achlorophyllous orchids mostly have mycorrhizal associations with homobasidiomycete fungi in the Cantharellales, Thelephorales, Agaricales, Serendipitaceae, Hymenochaetales, and Russulales, which are also pathogenic and ectomycorrhizal on higher plants [51]. In MH orchids, the fungi often form tripartite relationships, being ectomycorrhizal with woody plants and endomycorrhizal with orchids [23, 52, 53] where, transfer of carbon has been shown from the woody plants to the orchid [52, 54]. Fungal symbionts of MH orchids have three lifestyles: ectomycorrhizal (ECM), e.g. *Corallorhiza*-Russulaceae, parasitic (pathogenic), e.g. *Gastrodia*—*Armillaria* species, and saprophytic, e.g. *Epipogium*—*Coprinus* and *Psathyrella* species. Various achlorophyllous orchids such as *Gastrodia confusa* [55], *G. elata* [56], *Epipogium roseum* [57] and *Fulophis zollingeri* [58], are associated with many species of saprophytic wood- and litter- decaying fungi. Earlier studies have provided morphological and ultrastructural evidence that fungi from the Serendipitaceae formed ectomycorrhiza with *Corylus avellana* and *Carpinus betulus* [25] suggesting that common mycorrhizal networks (CMNs) are likely to be found in the plant communities where MH orchids are distributed in the close vicinity of ectomycorrhizal higher plants where they can obtain their nutrition through a tripartite relationship. Molecular studies have also shown the presence of *Serendipita* species on MH orchids such as *Hexalectris spicata* and *Neottia nidus avis*, suggesting that, if *Serendipita* is ubiquitous in its distribution, it is of interest to elucidate any functional symbiosis with ECM on higher plants.

Chlorophyllous orchids mostly have mycorrhizal associations with fungi in the *Rhizoctonia* alliance, in the Cantharellales and Sebaciniales (*Serendipita* Group B), with sexual stages in the Ceratobasidiaceae, Serendipitaceae and Tulasnellaceae [4]. Some of the *Rhizoctonia* species in the Ceratobasidiaceae are also plant pathogens of crops [4]. Fungal endophytes from the *Serendipita* group are common among photosynthetic orchids, e.g. *Caladenia* [42, 59] and non-photosynthetic terrestrial orchids, e.g. *Neottia* [53, 60, 61]. They constitute two major groups: A and B [36]. Group B forms mycorrhizae with green orchids while group A is generally associated with ECM and some non-photosynthetic orchids [26].

4. Fungal specificity

Fungal specificity is common in Australian terrestrial orchids [39, 62]. Taxonomically related groups of Australian terrestrial orchid genera are associated with taxonomically related groups of fungi. Both achlorophyllous and chlorophyllous orchid species can have fungal specificity [57, 63] but is more remarkable among heterotrophic orchid species [64]. By contrast, chlorophyllous photosynthetic mycorrhizal plants are said to be generalists in their associations with mycorrhizal fungi [4], though there is evidence of specificity at the species and strain level in Australian OMF and their host orchids, especially *Caladenia* [17, 65].

Most common genera of seasonally dormant terrestrial orchids in Australia belong to the Tribe Diurideae; within this, genera in the Sub-tribe Prasophyllinae usually associate with *Ceratobasidium*, those in the Caladeniinae with *Serendipita*, and most of those in the Diuridinae, Drakaeinae and Thelymitrinae associate with *Tulasnella*. Genera in the Acianthinae and the Megastylidinae associate with *Serendipita* and/or *Tulasnella*, e.g. *Thelymitra calospora* and *Lyperanthus nigricans* associated with a wide range of endophytes. Also, variations in seed germination rates with fungal isolates of *T. calospora* were noticed in *Diuris* species [39]. However, within these general relationships, fungal strain, seed and fungal provenance play an important role; specificity varies from high in *C. tentaculata*, in which seed and fungal provenance both varied seed germination significantly,

to low, in which more than one species of *Tulasnella* stimulated germination in *Thelymitra* [39].

OMF effectiveness leads to increased seed germination rate and fitness of orchids [66]. Specificity can be strictly restricted to the early seed germination stages of orchid or involve the compatibility of the fungal symbiont with the orchid throughout later stages [67]. Masuhara and Katsuya [62] has expanded fungal specificity into “potential and ecological specificity” whereas, earlier *in-situ* seed baiting studies from endangered and common orchids have shown distributions of OMF independent of their host orchids [68], suggesting that the patchiness of many orchids is not due to patchiness of their compatible species.

Previous research has also shown fungal specificity with particular orchid species during germination stages; for example, *Neottia nidus-avis* needs a specific *Serendipita*-like fungus to germinate [61]. Fungal specificity and effectiveness vary with individual isolates associated with the host orchid species for example, OMF isolated from *Caladenia* species were effective in germinating seeds of both *Caladenia* and *Glossodia* as compared to *Eriochilus cucullatus* and *Acianthus reniformis* [39]. These seed germination tests, under in-vitro conditions, over-estimate the potential of OMF isolates to form effective symbioses with orchid species, and results in a failure of symbiosis during later stages of orchid development thereby parasitizing the host plant [69]. Also, it does not explain the fungal switching that has been recorded during the lifetime of an orchid in the wild [70].

5. Nutritional trends in OMF

Decomposition of organic materials present in the form of dead decaying material such as fallen leaves, litter, hair, exoskeletons and any other kind of waste product from plants or animals is the main source of carbon compounds available.

In forest ecosystems, mineral nutrients in the form of P and N are mostly locked within living organisms or in the organic layer of soil. The distribution of these resources is heterogeneous in terms of space and time [71]. Access to nutrients by the host plant depends on the ability of the mycorrhizal fungi to mineralize the available organic nutrients to intermediate and soluble forms and then mobilize them to the host plant [72]. OMF can grow freely in the environment and have an ability to sustain itself without its host [21]. Mycelium is the predominant vegetative form among the basidiomycetes, comprising interconnected hyphae [71]. Fungal foraging for the uptake of minerals and other resources that are interlocked in the organic layer of the soil largely takes place at hyphal tips. Fungal hyphae have a large surface to volume ratios and secrete enzymes that digest extracellular organic resources, which are further translocated to a sink in the form of simple soluble compounds [73]. From the nutrient-deprived ecosystems of Australia, very limited information is available on the ability of OMF to utilize various C, N and P sources from the complex litter present on the forest floors.

For successful symbiotic interactions, efficient utilization of nutrients by the fungal partners is a prerequisite. In most mycorrhizal associations, photosynthetic products are transferred from an autotrophic host plant to a heterotrophic fungal partner, while the mineral nutrients obtained from the soil move in the opposite direction [74]. By contrast, in mycorrhizae of the photosynthetic orchids, the flow of nutrients is bidirectional, at least in some orchids [19]. In orchids, nutrient uptake into OMF occurs mainly through the acquisition of soluble nutrients from the decay of organic litter present in the top 4–12 cm of topsoil [73]. Information on the types of soluble carbon sources OMF can utilize from the environment and their host plants are very limited.

Few studies have reported inter- and intra- specific variations in utilization of substrates among orchid and ericoid mycorrhizal fungi from the same habitat [16, 18]. Also, Wright et al. [17] provided evidence of genetic and functional diversity among OMF isolates of *C. tentaculata* that varied in germination rates and utilization of some C and N sources. Unlike many ECM basidiomycetes, OMF has also retained the genes for the breakdown of these complex carbon compounds [31]. Understanding the nutritional roles of OMF may explain the diversity noticed among fungal isolates, from even single orchid plants in rates of symbiotic seed germination *in vitro*. However, in most cases, only one symbiotically effective fungus was examined from each orchid species from their habitat despite, a large number of fungal variations commonly isolated from even single plants. The symbiotic effectiveness of these isolates might vary with their ability to take up and utilize various carbon sources from their surroundings, an aspect that has not been studied so far.

5.1 Carbon sources: saprophytic ability of orchids and their dependence on mycorrhizal partners

During the early stages of orchid seed development, both achlorophyllous and fully autotrophic orchid species lack their ability to synthesize carbohydrates and the only available source of carbon and nitrogen to these plants is through OMF associated to them. One of the common assumptions so far in the orchid biology is that OMF can obtain its nutrition by digesting the litter components present on the forest floors and there has not been much evidence of their ability to grow on these litter components apart from few studies [15–18, 75]. There are reports where orchids are found in close vicinity of moss lying on the forest floors but there is no scientific evidence showing the presence of OMF on them or surrounding litter [15]. *S. vermifera* complex is mostly root biotrophic [37] and is associated with *Caladenia* species that is believed to be saprotrophic, at least as far as the fungi isolated from the Australian orchids is concerned.

In their natural habitat's orchids are commonly surrounded by litter such as bark, leaves and wood. During *ex-situ* measures for orchid conservation, these components have been extensively used as mulch in the pots of orchids to retain proper moisture levels. In Australia, Casuarina branchlets are commonly used as a source of mulch for re-emergence and growth of orchids during *ex-situ* conservation measures based on an assumption that they help orchid leaves from drying up but there is a possibility that these litter components on their break down may help them in the nutrition of the OMF and hence the orchid growth [75]. Recently, Mehra et al. [15] have validated their use in *ex-situ* cultivations by showing the amounts of fungal biomass produced on natural and semi-purified substrates from various endangered and common *Caladenia* species under *in-vitro* conditions.

5.1.1 Complex carbon sources in a litter

Nutrient-poor soils are inadequate in their microbial decomposition rates and the dead organic matter present on the soil is mostly utilized by decomposer fungi [76]. Litter constituting bark, wood, and leaves have biopolymers such as chitin, pectin, lignin, cellulose, hemicellulose and contain complex cell wall polysaccharides along with chitin of fungal and invertebrate origin. Most of this organic waste is in the form of plant cell wall components which constitutes 90% of plant cell wall components, having three major polysaccharides: cellulose, hemicelluloses and

pectin [77]. Of these, cellulose and pectin are key components of organic substrates in vegetation and are an important source of nutrients for ectomycorrhizal fungi [78]. Also, chitin is the main polysaccharide found in fungal cell walls and invertebrate exoskeletons [79] having significant quantities of nitrogen. These complex biopolymers are degraded enzymatically into simpler water-soluble forms of sugar through saprotrophic or mycorrhizal fungi reflecting their saprophytic ability which can be indirectly related to the survival of their host plant. For the survival of the host plant in wild, its nutritional demands for carbon and energy are met by the decomposition of this organic content present in the environment by OMF at the same site. Little information is available on the saprophytic behavior of OMF and more research is required to understand the nutritional physiology of both the partners by having a complete understanding of the role of OMF in decomposing the organic matter present in the ecosystem.

5.1.2 Litter degradation through enzymes

The decomposition of organic matter by saprotrophic basidiomycetes is a complex mechanism and does involve the participation of various enzymes and reactions. Saprophytic fungi stand apart from other organisms in their ability to decompose non-protein sources [73]. Various chlorophyllous and achlorophyllous orchid species are associated with saprophytic fungi from species of *Rhizoctonia* and *Epulorhiza* [80]. Utilization of these complex compounds in a litter is associated with the activity or production of extracellular enzymes (endo- or exo-) in basidiomyceteous fungi. These complex sources of carbon are degraded into their simpler forms through the activity of hydrolytic enzymes. Various litter components require a different set of enzymes for decomposition to occur such as cellobiohydrolases, Endo-1,4- β glucanases, and 1,4- β -glucosidases which effectively decompose cellulose to cellobiose. β -glucosidases then convert cellobiose to glucose.

Hemicelluloses are the second most abundant, heterogeneous polysaccharides present in the plant cell walls and comprise branched polymers of 500–3000 C5 or C6 sugars [81]. Lignin and plant cell wall polysaccharides (hemicellulose) interact with cellulose fibers to strengthen plant cell walls. Pectinases are widely produced by plant pathogens and endopolygalacturonase is one of the major enzymes involved in pathogenesis produced by a large number of pathogens such as *Rhizoctonia solani* [82], *Phytophthora infestans* and *Verticillium* species [83]. Several pathogenic fungi degrade pectin and the release of these enzymes allows them to infect their host plant under favorable conditions but activates the cascade of defense reactions in plant cells [84].

Recent studies on OMF from Australian orchids, in the genera *Caladenia*, *Diuris*, *Drakaea* and *Pterostylis*, have shown utilization of pectin as a sole carbon source, resulting in the production of fungal biomass ranging from greater than to less than that on xylan [16]. Several extracellular enzymes, such as dehydrogenases and oxidases from the mycelium, are involved in wood-lignin decomposition and have the potential to utilize all major constituents of litter [81]. Microbial decomposition in heathland soils is a slow process [85] and the penetration of the resource is important [86]. Most wood-associated decay reactions occur close to fungal hyphae due to limited amounts of diffused enzymes [81] and lignocellulose-degrading units in the cell walls [87]. Burnett [88] proposed that enzyme secretion may occur in different areas of the apical region and these findings were further supported by experimental evidence in *Neurospora crassa*, where structural and physiological differences in the hyphal cell wall at the apical region contributed to the variation in secretion and retention of exoenzymes in the wall.

5.1.3 Breakdown of complex sources into soluble compounds and their use

In many ecosystems, most of the nutrients are locked up in organic compounds, soil microflora and microfauna. Organic macromolecules present in the soil are degraded to intermediate forms through the saprophytic ability of decomposers adding up to higher decay rates in the soil [89]. Some of the complex compounds in the form of cellulose, hemicelluloses (xylans and arabinoxylans), starch and pectin are degraded to soluble intermediate forms such as oligosaccharides, disaccharides, cellobiose, xylobiose and maltose which are finally broken down to their soluble breakdown products such as glucose, mannitol, trehalose, arabinose, galactose, mannose, xylose, rhamnose and glucuronic acid.

On penetrating a substrate, fungi decompose it and absorb its nutrients. The available nutrients help the fungus to grow and proliferate until the nutrients are depleted and fungus becomes dormant. In nature, succession starts at this point and other species feed on the remains. Succession in microorganisms is very important in completely digesting complex carbon sources to simple soluble compounds. The C:N ratio plays a vital role in determining microbial growth and the amount of decomposition taking place. Inter-relationships are sometimes antagonistic, with exploitation, antibiosis and competition being very common [89]. An average of 30–40% of C from decomposed substratum is assimilated by the fungi under favorable conditions [89].

OMF, as saprophytes, break down these complex macromolecules and transfer the intermediate and final soluble products to their hosts. The fungal partner increases the efficiency of the host plant in acquiring C, N and P from litter and soil.

So, it is important to understand the ability of OMF to utilize soluble carbon sources. Fungi break down complex molecules into intermediate and then simpler water-soluble forms. These soluble forms are then assimilated and used in metabolic pathways, or liberated as free metabolites, to be used by the OMF or competitive microorganisms, and may be subsequently transferred to the host plants.

5.1.3.1 Use of soluble carbon sources by OMF

Some of the soluble compounds released on the digestion of complex carbon sources are simpler soluble forms of sugars in the form of monosaccharides and disaccharides. OMF vary in their absorption of nutrients from the soil, similar to other mycorrhizal fungi. The ability of OMF to utilize a range of soluble carbon compounds has been studied previously but information available is fragmentary if compared to other mycorrhizal groups such as ERM and ECM fungi. Earlier physiological studies have stated that OMF metabolize sugars through an activity of enzymes such as amylases and maltases and diastase-invertases [90]. There is little information available on the activity of enzymes and transporters involved in OM symbioses, but soluble carbon sources are likely to be transported rapidly to both pelotons and orchid cells which are later used in metabolism. Moreover, few studies have demonstrated the translocation and hydrolysis of the disaccharide sugar trehalose at the interface of the symbionts in MH orchids [91, 92]. Isotopic studies have shown a two-way transfer of carbon between the OMF and the orchid host [91, 93, 94] and it has recently been suggested that C and N containing compounds (derived from glucose and ammonium nitrate) are transferred from both senescent and live pelotons in *Spiranthes sinensis*–*Ceratobasidium* sp. AG-1 symbiosis *in vitro* [95].

To understand the potential of OMF to use soluble carbon sources requires their growth on a range of single carbon sources followed by measurement of their

growth as fungal biomass. Research on Australian OMF has generally shown utilization of various soluble carbon sources such as the C5 arabinose, C6 glucose, C12 sucrose and cellobiose, and C(n) cellulose (as CMC), xylan, and pectin, and tannic acid [16–18]. Biomass on soluble carbon sources can be easily quantified by measuring the dry weight of mycelium and subtracting the biomass of controls from all the treatments, as used by Midgley et al. [16], Wright et al. [17] and Nurfadilah et al. [18] and Mehra et al. [75]. More recent studies have shown trends in utilization patterns of carbon sources across four fungal taxa from the *Rhizoctonia* alliance (*Ceratobasidium*, *Rhizoctonia*, *Tulasnella*, and *Serendipita*). OMF from these taxa produced large biomass on xylan, glucose, cellobiose, cellulose, pectin, and to some extent CMC, and the least fungal biomass was reported in all for tannic acid [18]. In studies on OMF from Australian orchids in the genera *Caladenia*, *Diuris*, *Drakaea* and *Pterostylis*, xylan consistently produced the greatest growth, often exceeding that on glucose [16–18].

For the establishment of balanced symbiosis between two partners more research using similar methods is required to determine the nutritional preferences displayed by OMF from other Australian terrestrial orchid species. The ability of an OMF to compete for and use soluble carbon compounds from sources external to the orchid may reflect the ability of its host orchid to survive and thrive.

5.2 Nitrogen sources

N present in the soil litter is typically found in the form of inorganic N (nitrates and ammonium) and organic N. Organic N comprises a large fraction of Australian litter but its utilization by OMF has been poorly studied. In the natural environment, amides and amino acids are easily accessible to the OMF, external to the orchid as a result of a litter breakdown and internally in the orchid as a result of plant metabolism.

The utilization of a wide range of organic and inorganic forms of nitrogen by OMF suggests their specificity of enzymes to hydrolyze complex forms of amides and peptides into simpler soluble organic N sources that are directly absorbed by OMF. The uptake and transfer of N by OMF has already been reported previously for northern hemisphere OMF [20, 96, 97] whereas, Cameron et al. [93] provided direct evidence of uptake and transfer of organic N through *Ceratobasidium cornigerum* (from *Goodyera repens*) by double-labeling of amino acid glycine. Recently, studies have also shown a transfer of N from the soil and through tripartite relationships by a single OMF of MH orchid, *Rhizanthella gardneri* [98]. Most recently, the uptake and transport of nitrogen from NH_4NO_3 was inferred from isotopic enrichment of ^{15}N in the pelotons and uninfected cells of *Spiranthes sinensis* protocorms using ultra-high spatial resolution secondary ion mass spectrometry (SIMS) [95]. With inorganic N sources, most authors reported greater utilization of NH_4^+ than NO_3^- in OMF strains of *Tulasnella* (one strain, *C. flava*) and *Serendipita* (six strains, *C. tentaculata*) whereas, many of these did not utilize nitrate [17, 18]. With organic sources, most OMF were capable of utilizing C3 alanine, C4 aspartic acid and/or asparagine, C5 glutamic acid and C6 arginine well as compared to C5 proline and C6 histidine which were poorly utilized [17, 18, 99]. Few OMF utilized C2 glycine well and others poorly; the latter included an isolate from *C. flava* [18]. In addition, only two out of six OMF from Australian *Pterostylis* species utilized tryptophan [16]. Recently, research work on Australian endangered orchid species (*C. fulva*) has shown that one of the symbiotically effective isolates, utilized most of the N sources with minimal variations in their biomass in contrary to the ineffective isolate under *in-vitro* conditions. The reason suggested for this was that it would affect their competition, at both levels in the host plant (internal/external) whereby, an ineffective

isolate can successfully outcompete the effective isolate and its host, leading to chlorosis before the death of an earlier surviving orchid seedling [15].

5.3 Phosphorus sources

Most Australian soils are ancient and are phosphorus-deprived, as most of it has been leached out over time [100]. Along with N, it is one of the major limiting factors for plant growth. In soil, it is present in two major forms: inorganic P (P_i) in the form of phosphates where they are present in the form of scarcely available complexes [101] and mineral and organic phosphorus (P_o) as phosphate diesters, phosphate monoesters and inositol phosphates [100] where they are low in orthophosphate levels [102]. In natural environments, fungi degrade organic phosphorus compounds present in the dead matter but organic phosphorus locked in humus-rich forest soils is not easily accessible [100, 103]. Inorganic phosphorus has low solubility and is present in three main fractions: soil solution (dissolved phosphates), a labile pool (phosphates adsorbed to surfaces) and a non-labile pool (metal phosphates) [104].

Plants cannot utilize organic phosphates as they only have access to soluble phosphates and can readily absorb them [104]. Mycorrhizal associations can overcome nutrient limitations to plant growth by increasing the availability of phosphorus. Fungi can release phosphorus into the soil solution from organic phosphates with the help of phosphatases, thereby providing access for plants to otherwise insoluble forms of phosphorus [105]. The greater availability of phosphorus to the mycorrhizal plant host is dependent on the ability of its symbiont to absorb and translocate inorganic phosphates to the host roots and to access the forms of phosphorus 'locked up' in organic debris [106, 107]. Fungi can store phosphorus in their vacuoles as polyphosphate chains or as condensed phosphate [108].

Terrestrial orchid habitats are nutrient-deprived in Australia and leaf litter is among one of the major phosphorus sources available to OMF [100], through its richness in the cyclic phytic acid (inositol hexaphosphate, IP6, inositol polyphosphate), the main form of phosphorus storage in plants. In orchids it is assumed that mycorrhizal associations benefit the host plant by increasing the uptake of phosphorus. Earlier studies have reported the secretion of acid phosphatases by fungi in pure cultures [43]. The transfer of organic phosphorus in young protocorms of orchids through mycorrhizal fungi was first demonstrated by Smith [109] whereas, the uptake of inorganic phosphorus in mycorrhizal adult seedlings of *Goodyera repens* has been reported previously. Whilst, the utilization of organic phosphorus was demonstrated by Smith and Read [4] through the hydrolysis of organic compounds with a release of inorganic phosphorus (P_i). So far, there are few studies on the utilization of various forms of phosphorus by OMF in contrast to extensive work done on other mycorrhizae. A recent study by Nurfadilah et al. [18] showed that OMF from four genera of Australian orchids produced greater biomass with inorganic phosphate than DNA and with intermediate levels in case of phytic acid.

6. Ecological implications

Fungal preferences for specific carbon sources from the heterogeneous and unstable distribution of the substrates on forest floors might suggest that different stages of host plant development may have a preference for different organic substrates, for example, the abundance and presence of orchid seedlings (*Tipularia discolor*) near decaying logs in specified habitats as opposed to their absence near-adult flowering individuals [43] suggests that OMF does have preferences for their carbon

sources, which could therefore explain their patchy distribution in the environment. The relative lack of utilization of some soluble components likely to be generated, may offer opportunities and niches for other fungi and microorganisms in general. OMF must compete not only with one another but also with other mycorrhizal and saprophytic fungi for these resources, and for their breakdown products.

The relative abilities of OMF from Australian endangered and common orchid species (*Caladenia* spp.) to grow on the breakdown products of litter may have some ecological implications for their orchid hosts in terms of their taxonomy and conservation status [75]. Similarly, Nurfadilah et al. [18] concluded that the OMF from rare and common orchid species has the same utilization profiles of soluble carbon sources, having slow and uncompetitive growth could explain the conservation status of its host orchid. The importance of these nutritional studies can be related to the patchy spatial distribution of OMF and their host orchids [110]. Previous *in-vitro* studies showed competition between orchid siblings for available resources through their OMF and there is a possibility that this could be true for the orchids growing in the wild [111, 112].

Mehra et al. [75] showed that the OMF from various *Caladenia* species are differentiated not so much by different profiles of carbon sources utilized but by different rates of growth and final biomass. This suggests that threatened orchids contain OMF with relatively slow-growing and uncompetitive OMF compared with those from common orchids. It would be interesting to test this further by examining more OMF from a greater range of orchids. Also, *Ceratobasidium* species have rapid rates of growth compared with those of *Serendipita* and *Tulasnella*, the other two main OMF of Australian orchids, and it would be interesting to test these in direct competition in microcosms to see the effects on the survival of orchid seedlings of their respective hosts.

7. Conclusion

Orchids depend on their fungal endophytes for their nutritional demands, which is obligatory in its initial stages but may vary in adult green orchids, though they continue to harbor OMF in their underground organs. The forms of C, N, and P available to the OMF can determine their availability to the orchid host and can indirectly affect its conservation status. Thus, obtaining an effective symbiont is critical for an orchid's survival and is absolutely a high priority in recovery plans for endangered species. In order to develop effective strategies for conservation of orchids, a large number of orchid taxa should be tested for their nutritional modes as a function of their habitat based partly on organic content using labeling techniques and isotopic fractionations. Also, future research should be focused on developing enzymatic profiles for OMF using sterilized natural substrates and insoluble carbon sources, which may augment our understanding of the role of OMF in the decomposition of organic matter in the ecosystem. Uptake of soluble carbon sources in OMF from terrestrial green orchids can be further investigated through radiotracer techniques through labeling and setting up small microcosm experiments. Tracing the translocation of external highly enriched carbon sources over a short period of time will provide evidence on the net transfers of different forms of carbon between the OMF and the orchid.

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